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ANTIBIOSIS[1]

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The term antibiosis, which is the title of this paper, may to many biologists be a rather new term that has been in frequent use only since the discovery of penicillin in 1929. But in reality it is a term used by botanists for a long time. It seems to have been coined by H. M. Ward (1) in an article on Symbiosis published in Annals of Botany in 1899. The more familiar term "symbiosis" has long denoted the partnership of two organisms, often of different type, for their mutual benefit. An example is the common root tubercle bacteria which live in the nodules on the roots of peas, clover, etc. Here the bacterial cells get a favorable place to live and in turn their work of storing up compounds of nitrogen, later enriches the habitats of their hosts. In the article referred to above Ward said, "Where one of two associated organisms is injuring the other . . . this state of affairs has been termed antibiosis."

This harmful relationship was studied more thoroughly by the German writer, J. Behrens (2), in 1904 in a paper entitled "Alteration Effects between Different Organisms," which appeared in the Handbook for Technical Mycology at Jena. In this article Behrens discussed symbiosis, metabiosis and *antagonism* between organisms. Dr. Erwin F. Smith (3) of the U. S. Department of Agriculture in 1905 published studies along similar lines in his article, "Bacteria in Relation to Plant Diseases." The earliest detailed work done on this subject was done in the 1890's by a French bacteriologist, C. Bouchard, and others in Europe. The entire literature in the field has recently been surveyed by Dr. Selman A. Waksman (4), the discoverer of streptomycin, who has been extremely kind and helpful to me in the preparation of this paper. He and his colleagues at Rutgers have reviewed over 1,000 articles dealing with antibiosis and I shall quote briefly the chief references in this early work. "Bouchard (5), Emmerich and Low demonstrated that certain bacteria belonging to the *Bacillus pyocyaneus* group have the capacity of inhibiting the growth of other bacteria and of actually killing these organisms. This was found to be due to the production of a substance designated as *pyocyanase*, the first antibiotic substance ever described. However, this phenomenon was looked upon rather as a freak, the substance being regarded as an enzyme that had the capacity of bringing about the lysis of certain bacterial cells. The plant pathologists constantly observed in their cultures of fungi and bacteria, the detrimental action of one organism upon another, but they were satisfied to designate this phenomenon as one of "staling" without attempting to uncover the manifold reactions involved. The soil microbiologist, in his studies of mixed populations of micro-organisms, long recognized the fact that one organism may affect, to a marked degree, the activities of another, but he dealt with such a highly heterogenous medium, the soil, that his problem appeared

[1]Presidential address, delivered before the Ohio Academy of Science at Marietta, Ohio, May 2, 1947.

hopelessly complex. By designating as "soil toxins" the injurious substances thus produced he failed to succeed in unravelling further the complicated reactions involved. The chemist isolated and even crystallized certain antimicrobial substances produced by bacteria, such as *pyocyanin* and by fungi such as *penicillic acid* and *gliotoxin*, but even then the practical utilization of these substances was hardly comprehended. Only isolated investigators recognized the great possibilities involved in the antagonistic inter-relations among micro-organisms and drew attention to their possible applications. This was first done by Pasteur and Joubert in 1877, followed by Cantani in 1885, by Bouchard, Emmerich and Low in 1889, by Vaudremer in 1913, by Much in 1924, by Fleming in 1929 and finally by Dubos in 1939. It remained for the last half decade to uncover the great potentialities of this highly important field of microbiology with its many phases touching upon Chemistry, Physiology, and Chemotherapy." In concluding this review of the history of antibiotics, I might say that the last named student in this field, Dr. R. J. Dubos, a colleague of Waksman's at Rutgers and now at Harvard, has in the period between 1929 and 1944 published 19 papers, and Dr. Waksman himself, 38 papers, on this subject in various medical, chemical, and biological journals. This indicates the great activity in this field today.

ORGANISMS IN THE SOIL AND THEIR INTERRELATIONS

Since most of the antibiotic substances in use today as therapeutic agents have been isolated from organisms which inhabit soil, it is now appropriate to examine this complex thin layer of the earth's surface.

Waksman and his associates have made thousands of analyses of soil, both biologically and chemically. Dr. Charles Thom (6), a specialist on molds in the U. S. Department of Agriculture, has said, "a teaspoonful of soil contains hundreds of millions of bacteria, millions of actinomycetes, hundreds of thousands of mold spores, tens of thousands of protozoa and hundreds of nematode worms. In such an environment a delicate, soft-walled plant like *Penicillium notatum* needs protection comparable to a tank in human warfare."

Looking first at the chemical composition of the soil it is chiefly a mass of inorganic particles of sand, clay, and pulverized rock, which constitutes 90 to 99 per cent of its volume. The organic matter in the top layer of soil varies from 1 to 10 per cent, although in dry desert and sandy soils the organic part may be less than 1 per cent and in peat and muck soils it may be increased to 50 or even 90 per cent. Contrary to what one might expect the organic or humus part of the soil is not mainly cellulose or hemicellulose which occurs in plant bodies but is high in lignin and protein content. These substances are less amenable to microbial action than the actual bodies of plants and animals. To a large degree this black mixture is soluble in alkalies and can be reprecipitated by acids. Collectively these substances have been termed "humic acids." The chief mineral components of average soils are the phosphates, sulfates and silicates of calcium, magnesium, potassium, iron, aluminum, manganese, zinc, copper and others. Some of the chemical elements derived from these minerals are utilized by organisms to make protoplasm and food such as nitrogen, sulfur and phosphorus. Others as zinc, copper, iron, manganese, boron and even potassium may serve as catalysts in life processes. The functions of these latter elements are as yet little known in the metabolism of living things. The top region of the soil in which the root systems of higher plants live is called the rhizosphere and it is in this layer that most of the biological activity of the soil occurs. It is here that the millions of organisms live as mentioned by Dr. Thom. Most of these microbial organisms are supported by the wastes or debris of the bodies of higher plants and animals that live on the surface of the earth. Some of the minute forms of life, living in the soil, are also dangerous enemies of the higher organisms that are on the surface. These are

exemplified by the root-rots, blights and wilts that attack crop plants as well as bacteria and fungi that can attack animals and men as the tetanus and botulism organisms, pathogenic yeasts and molds. Certain pathogens which normally live only in the bodies of higher animals may temporarily live in the soil having been deposited there with wastes. These may later reinfect other animals. Waksman classified the microbial denizens of the soil into eight groups, viz.: bacteria,

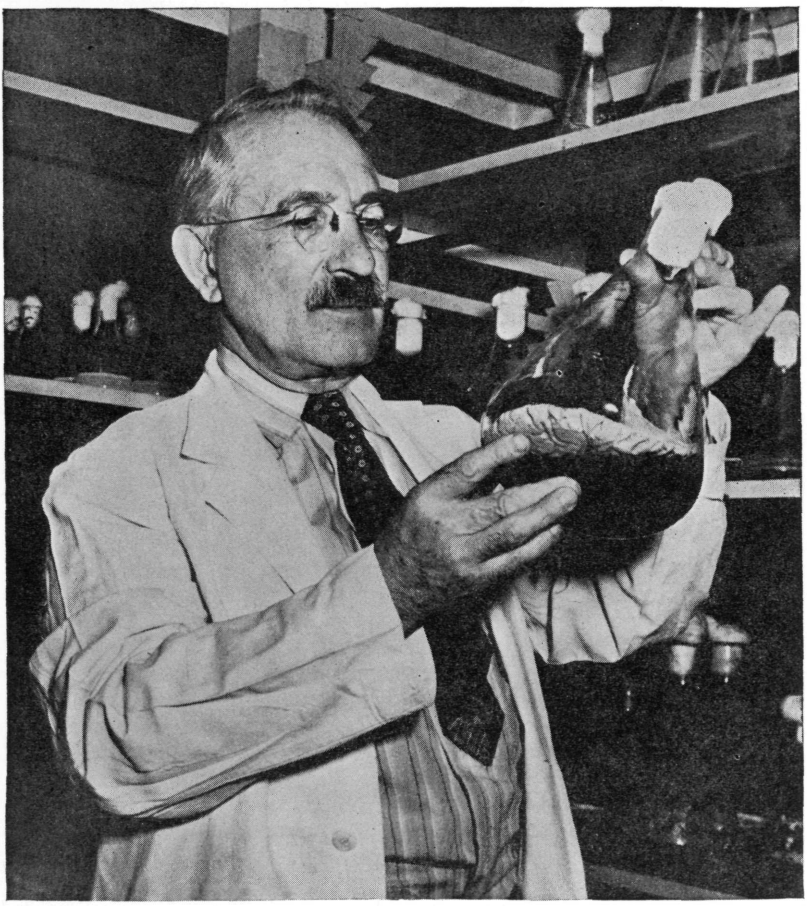

FIG. 1. Dr. Selman A. Waksman.

actinomycetes, fungi, algae, protozoa, worms, insects and near-microscopic forms, and ultra-microscopic forms such as viruses and phages.

The relative abundance of the above organisms is dependent on the nature of the soil, the amount of organic matter in it, the oxygen supply, the moisture content, the temperature, and the buffer capacity—and also the type of the higher plants growing in the area. The bacteria, actinomycetes and true fungi run into the thousands per gram of soil. Algae are numerous only in the surface layer of soil. Protozoa are found in the active state or in the form of dormant cysts. This depends on the moisture content. Sometimes the protozoa only are in the

active stage for a few hours during excessive moisture while the encysted forms occur almost uniformly in dry soils. The commonest protozoa in soil are the flagellates which may reach a population of a million individuals per gram of soil, the amebae are next in abundance and the ciliates, the fewest, numbering a few hundred to several thousand per gram of soil. The nematode worms, rotifers, earthworms and insect larvae are usually abundant.

The bacteria are of various types based on their nutrition, respiration and motility. They may be autotrophic, heterotrophic, aerobic or anaerobic,motile and non-motile, pathogenic and saprophytic, symbiotic and non-symbiotic, antagonistic and non-antagonistic. The fungi are classified into three types: saprophytic and free-living, mycorhiza-producing, and plant pathogenic. The commonest genera of soil fungi are these: *Rhizopus*, *Mucor*, *Penicillium*, *Aspergillus*, *Trichoderma*, *Fusarium*, *Cladosporium* and *Cephalosporium*. There are also numerous yeasts and fleshy or mushroom types of fungi. The mycelia of these latter fungi spread extensively through the soil and hold it together in masses.

It is now recognized that all the higher plants and animals suffer from the ravages of microbes such as bacteria, fungi and protozoa; and the more highly evolved the plant or animal, the more numerous are its microbial parasites. Many of these disease-producing agents are closely related to harmless forms which lead independent lives in soil or water. This leads to the assumption that the disease-producing agents were once non-pathogenic but which became adapted to a parasitic life in specific hosts. In such hosts these agents produce toxic substances which injure the host for a greater or shorter period until the host succeeds in building up a resistance to the pathogen or it is finally killed by it. If the host survives the attack, however, without killing its parasite, it may regain a normal life but is henceforth a carrier of the pathogen. These disease-producing organisms spread from host to host through natural media as water, dust or excreta of the host and so reach the soil or water.

When one takes into account the great length of time that plants and animals have lived on the earth and the millions upon millions of disease-producing organisms which caused their deaths and then passed into the soil and water with their disintegrating host, it would be logical to conclude that the soil and water of the earth are the great repositories of disease-producing microbes. This was the opinion of the scientists of the 19th century. But careful investigation of soils and waters show that only a few forms which would attack men or animals live in these media. It was furthermore found that pathogenic organisms that kill their hosts, when followed directly into the soil or water, do not live long and disappear rapidly. Researches on soil showed that if it is actually sterilized and then some pathogens inoculated into it, they may flourish there, but if these same pathogens are planted in fresh soil, they die quickly. Thus it is seen that the natural soil and water of the earth are not great repositories of disease-producing organisms but that just the opposite prevails. Ordinary soil by reason of its own normal microbial population destroys any pathogens which find their way into it. It has been found that even the common *Rhizobium*, a bacillus that forms nodules on the roots of leguminous plants, cannot thrive outside the nodules of the roots. Waksman (7) found that the normal soil microbes will quickly kill it when it is placed alone in soil. It survives only when protected in the security of its nodules on the plants. Another instance is the case of the Texas Cattle Fever organism. This lives in pastures of the southern states. Smith and Kilbourne (8) showed this organism to be a protozoan which is inoculated into the cattle by a tick which is a carrier for the parasite. This protozoan is not able to live in climates which are cold enough to kill the ticks. Thus it is seen that it cannot survive in the soil of the pasture unless it is protected in the body of the tick.

ORGANISMS PATHOGENIC FOR MAN

Briefly at this place let us pause to look at the micro-organisms which may produce diseases in man. These small forms are found in both the plant and animal kingdoms. In the latter belong the Protozoan parasites which infect man inside his body or on the surface. In this group fall the amebae, which live in his intestine or in the mouth, the malaria which devour his red corpuscles, the flagellates which may live in the genital tract or the trypanosomes that cause sleeping sickness in nervous tissues and the ciliate, *Balantidium,* that infects the colon. But by far the greater number of his ills are due to plants which are parasitic members of that great group comprised under the vague term of Mycetes or Fungi. This poorly

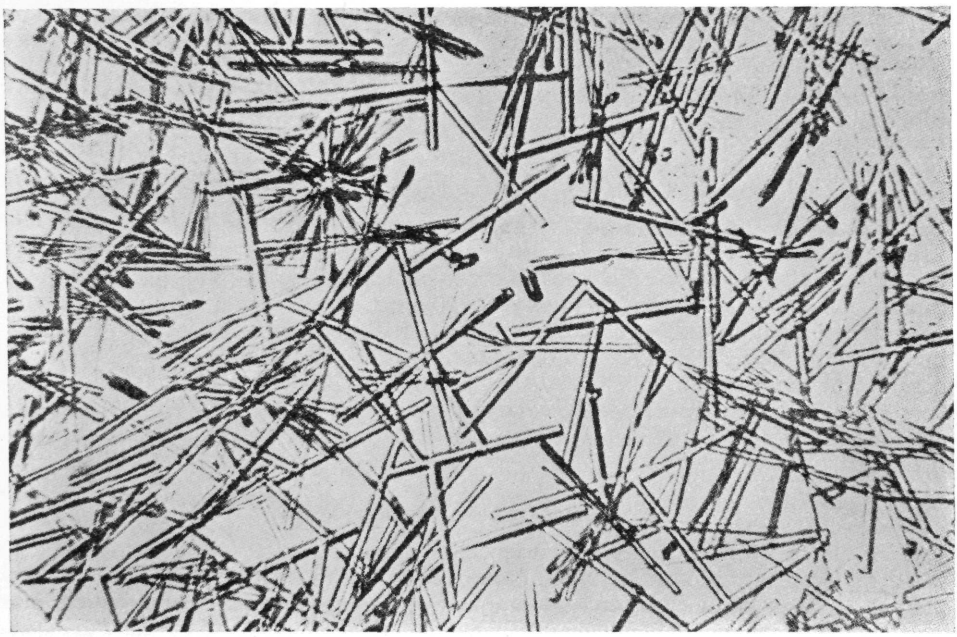

FIG. 2. Streptomycin crystals. (× 350.)

defined and mixed aggregation includes the Schizomycetes or Fission Fungi and the Eumycetes or True Fungi. The Schizomycetes are made up of three smaller groups; 1, the Eubacteriales or True Bacteria; 2, the Spirochaetales; and 3, the Actinomycetales. The True Bacteria are now pretty well defined and contain most of the familiar bacilli, cocci, and spirilla. The latter contains the *Vibrio* that causes Asiatic Cholera. The second group, Spirochaetales, may have descended from protozoa. It includes the Syphilis organism, *Treponema.* The third group, Actinomycetales, is supposed to have descended from the true fungi, are called the Higher Bacteria. One division of it—the Mycobacteria—includes the organisms that produce tuberculosis, leprosy, and diphtheria. The other division of the Actinomycetales consists of harmless soil organisms, some of which furnish antibiotics like streptomycin.

Associated with the True Bacteria are the Rickettsias, which seem to be very small forms that may have evolved from regular bacteria and have become pasasites such as cause Typhus Fever and Rocky Mountain Spotted Fever. Besides these there is the large group of viruses. These are so small they can pass through

fine porcelain filters and bacterologists have not determined whether they themselves are organisms or some product of organisms like enzymes. They differ in character from bacteria and fungi but are definite entities which produce such diseases in man as rabies, smallpox, yellow fever, parrot fever, chickenpox, German measles, mumps, infantile paralysis, influenza and common colds, and herpes or cold sores. These viruses are smaller than rickettsias and cannot be grown on culture media unless living cells are also present. They multiply only in young living susceptible cells, where they live like intracellular parasites. Their presence is indicated by tiny particles of uniform size in the cytoplasm of the cells. These are called inclusion bodies of virus bodies and have been isolated by centrifugation and studied in the production of vaccines.

Before leaving the subject of classification of pathogenic organisms it will be well to mention an artificial classification in the true Bacteria which divides them all into two important physiological classes. These two artificial groups are formed by a simple staining technic used by bacteriologists called Gram's Stain. A dried film of bacteria on a glass slide is stained with a violet analine dye, next this is treated with a solution of iodine and iodide. The dye is removed by immersion in alcohol to a state colorless to the naked eye. It is washed with water and counterstained with a pink or brown dye. Upon examination under a microscope the bacterial cells will appear either violet due to the first stain or pink or brown with the counterstain. The difference in this staining behavior in different types of bacteria is due to the peculiar composition of the protoplasm of the cell and its interaction with the reagents employed. Bacteria of a protoplasmic type which retain the violet stain are said to be Gram-positive and those which do not hold it but instead take up the pink or brown counter stain are called Gram-negative. In subsequent discussion of antibiotics there is a sharp distinction in their reactions to these substances by gram-positive and gram-negative bacteria. Some antibiotics are antagonistic to only one of the two groups of bacteria and some can inhibit both sorts.

THE NATURE OF ANTIBIOTICS

The various chemical compounds called antibiotics have certain properties which differentiate them from common antiseptics and disinfectants. In the first place, they are produced by living organisms, although recently penicillin has been synthesized directly from non-living chemicals. Antibiotics in general may have merely an inhibitory effect on the growth of bacteria or they may actually destroy them by a complete dissolution called lysis. Waksman classifies antibiotics in the broad sense as any substance which may produce the two effects above mentioned on bacteria. These antibiotics may be products of higher plants, like quinine from the Chinchona tree, certain proteins of wheat flour, the oils in onions and garlic which kill not only bacteria but as Professor B. P. Tokin (9) of Russia showed in 1944 may kill higher organisms like yeasts. He calls these antibiotics *phytoncides*. Likewise in 1944 Cavallito (10) and Baily of Rensselaer, New York, showed that the juices of burdock, butter-cup, dog-tooth violet and wild ginger, all contained similar antibiotic substances. These men showed also that the amino acid, cystine, inactivated these phytoncides and also the antibiotics derived from the lower organisms such as molds, like penicillin and streptomycin. In January, 1947, Dr. Alfred Marshak of the U. S. Public Health Service, reported an antibiotic for the tuberculosis organism derived from an epiphytic lichen which grows in California, *Ramalina reticulata,* called Spanish Moss. Its yellow crystals retard tuberculosis in experimental animals and appears to be non-toxic. Waksman includes in the broad sense antibiotics derived from animals such as lactenin and lysozyme found in tears and perspiration. But more specifically the therapeutic antibiotics refer to the antagonistic substances produced by micro-organisms. In 1903 Krenckner

(11) noticed that the culture filtrates of bacteria inhibited the growth of bacteria. It is now known that certain bacteria, fungi and actinomyces do produce these substances. These antibiotics are very selective in their action. They affect chiefly the gram-positive bacteria and to a lesser extent the gram-negative bacteria and a few antibiotics can inhibit the growth of both types of bacteria—each antibiotic thus has its own bacteriostatic spectrum. Their production is determined by the strain of the organism, the composition of the culture medium, the temperature of incubation, the age of the culture and the aeration. The antibiotics vary greatly in their action on bacteria, their toxicity to the animal in which the

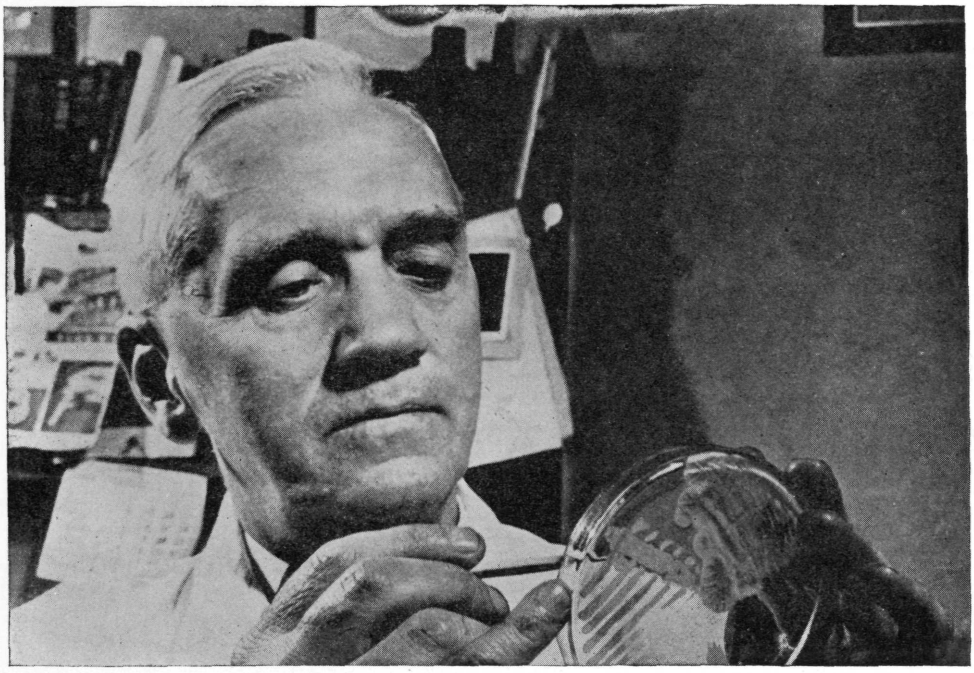

FIG. 3. Sir Alexander Fleming.

bacteria live and in their practical uses in treating disease. Thirty-seven or more of these antibiotics have been isolated and studied for their chemical properties and their biological activities. A few of the commoner ones are discussed here. *Actinomycetin* from *Streptomyces albus* is soluble in water but precipitated by alcohol, is thermolabile and protein in nature; it is lytic to dead gram-negative bacteria and to some living gram-positive forms. On the other hand, *actinomycin* is secured from *Streptomyces antibioticus*. It is soluble in ether and alcohol, insoluble in petrol ether; orange colored; thermolabile and is lytic for gram-positive bacteria and in higher concentration for gram-negative bacteria and is highly toxic to animals. Here then are two antibiotics from closely related soil organisms but with quite different properties. *Aspergillic acid* is derived from *Aspergillus flavus*, a fairly common mold. It is soluble in water, alcohol, ether and acetone but insoluble in petrol ether. It is acid in nature and is lytic to both gram-positive and gram-negative bacteria. Another antibiotic is *clavicin*, *clavitin* or *patulin* which is formed in several species each of the molds *Aspergillus* and *Penicillium*. It is soluble in ether, chloroform, alcohol and water. It is lytic to both gram-positive

and gram-negative bacteria as well as fungi. It is highly bactericidal but is toxic to animals. *Flavicin, flavitin, aspergillin* or *parasitin* is a substance produced by three different species of the mold *Aspergillus* and in all its characteristics is similar to the antibioltic *penicillin. Gramicidin* is an antibiotic isolated from *Bacillus brevis.* It is a polypeptide and is thermolabile; soluble in ether and alcohol; lytic to gram-positive bacteria but is hemolytic in the bodies of animals. The common mold *Penicillium,* in at least four or five species, produces several antibiotics. The most famous is *penicillin.* Others are *penicillic acid. penicidin, notatin* and *mycoin. Penicillin* is isolated from *Penicillium notatum;* soluble in alcohol and water and in ether at pH 2; thermolabile and lytic to gram-positive aerobic and anaerobic bacteria and of low toxicity in animals. Still other antibiotics are *streptomycin* and *streptothricin,* both derived from two different species of *Streptomyces,* an Actinomycete genus. They are both soluble in water and in acid alcohol, insoluble in ether. They behave like organic bases and are thermostable. *Streptomycin* is lytic to gram-negative bacteria and of low toxicity in the bodies of animals. *Streptothricin* is lytic to gram-negative and some gram-positive bacteria and has a higher toxicity in animals. *Bacitracin* (12) is another new antibiotic isolated from strains of *Bacillus subtilis.* It is water soluble, thermostable and acid stable, non-hemolytic, non-stable in alkali, resists digestion by pepsin and trypsin and is non-toxic in animals. *Subtilin,* also derived from *Bacillus subtilis,* is soluble in alcohol and is stable to heat and acid but unstable in alkali. It is lytic to both gram-positive and gram-negative bacteria and slightly toxic to animals. *Chlorellin* (13) is an antibiotic derived from a green alga, *Chlorella vulgaris.* It is antagonistic to the same groups of organisms that are susceptible to penicillin but has the added advantage that the alga is much simpler to cultivate than fungi, needing only water, a few mineral salts and an adequate amount of carbon dioxide blown through the culture. Of all the thirty-seven or more antibiotics discovered, only *penicillin* and *streptomycin* have come into wide general use and to a lesser extent *bacitracin* and *chlorellin.*

We will now discuss the discovery and manufacture of these two new and dramatic drugs.

First I should like to relate an example of natural antibiosis that occurred in my own observation. About four years ago in Toledo a friend of mine was troubled with an infection of "athlete's foot" fungus on his leg. He consulted a dermatologist who treated it with drugs and X-ray therapy but the infection was slow to heal. After several months, my friend had the misfortune to break his leg in a traffic accident and was hospitalized. While in the hospital he contracted erysipelas severely on his back and legs. This was treated by routine methods and in a short time was cured but the "athlete's foot" fungus which had been living in the area of skin attacked by the erysipelas had also entirely disappeared. This was a natural case of antibiosis in which the *Streptococcus* organism of erysipelas had destroyed the fungus *Trichopyton* which caused the "athlete's foot" disease.

The discovery of penicillin occurred in 1929 by Dr. Alexander Fleming, now Sir Alexander Fleming, while working at St. Mary's Hospital in London with some culture plates of *Staphylococcus aureus.* He noticed that in a plate which had become contaminated by a mold spore, the area around the mold mycelium showed a clear zone where the *Staphylococcus* had disappeared. He cultured some of this mold in broth to see if it had really killed the bacteria. In later experiments he found that if he took a drop of the broth in which the mold, identified as *Penicillium notatum,* had grown and placed it on a living culture of *Staphylococcus,* as the broth spread over and through the agar on the plate, it would kill off the bacteria. Dr. Fleming discovered that several varieties of *Staphylococcus* were susceptible to the substance in the broth and still later he learned that the bacteria causing boils, diphtheria and abscesses with pus formation could be entirely inhibited by the

broth in which the mold *Penicillium* had grown. On the other hand, he learned that the colon bacillus from the normal intestine and organisms which cause typhoid, dysentery and influenza were not affected at all by the broth substance.

Next he injected the broth into animals and found that they were no more irritated than if pure sterile broth had been used. In the British Journal of Experimental Pathology (14) in 1929 he modestly suggested that perhaps this antibacterial substance in the broth might be used clinically to help infections caused by the bacteria which he found susceptible to it. Since the mold which produced the antibiotic was *Penicillium*, he called the substance *penicillin*. For ten years this antibiotic excited little interest in the world: First, because it was a very unstable

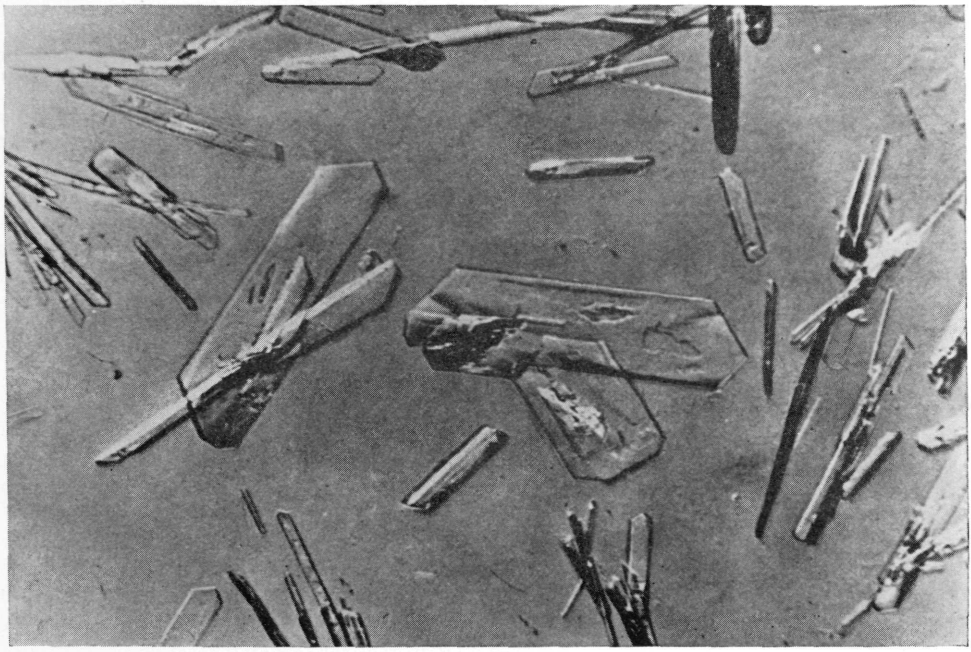

FIG. 4. Penicillin crystals. (× 500.)

substance and second, it could be produced only in very small amounts. During these ten years Dr. Fleming wrote several articles in which he used penicillin to differentiate various strains of bacteria. In 1931 Clutterbuck (15) and others told in the British Biochemical Journal that they were successful in growing the mold in larger amounts and had worked out the features of penicillin chemically. In 1943, Dr. H. W. Florey (16) and others in the "Lancet" described the use of penicillin as very effective in checking infections and that it had a low toxicity in the human body.

Just prior to this, in 1939, Dr. R. J. Dubos (17) in the Journal of Experimental Medicine announced the isolation of some bacteriocidal fractions from the culture media in which had been grown the soil organism *Bacillus brevis*. These substances were classified as polypeptides and were named *gramicidin* and *tyrocidin*. They were clear colorless crystals and were antagonistic to a number of bacteria but clinically they were not useful on account of the relatively high toxicity to the patient.

In 1940 and 1941 Florey and his co-workers reported in some 300 papers their results in treating several hundred patients in England with penicillin. In 1941 the interest in the drug became general and Professor Florey was invited to this country by the Rockefeller Institute to attempt a large-scale production of penicillin which was then impossible in England due to the bombing and devastation of the war. Our National Research Council and the Northern Regional Laboratory of the U. S. Department of Agriculture at Peoria, Illinois, and several large pharmaceutical firms all co-operated to make large-scale production possible.

At first the yield of penicillin was only two units per milliliter of culture medium. This was not fast enough since a million units are necessary to treat a deep seated infection. The next important step was the discovery by Dr. Andrew Moyer at the Northern Regional Laboratory that a by-product of the corn refining industry, called corn-steep water could be used as an ingredient in the culture medium in which to grow the mold. When this was added, it increased the yield of penicillin to more than forty units per milliliter of fluid.

All penicillin is now produced from a highly selected strain of *Penicillium notatum*. This mold grows most anywhere on decaying vegetables, dairy products or in the soil but a strain in a special collection called the Type Culture Collection in Washington, D. C., is labeled N. R. R. L. 832. This was found growing on a piece of decaying cantalope. This strain is the best known producer of penicillin. Penicillin made from unknown or wild strains of *Penicillium* have been found to induce severe anaphylactic symptoms and allergy in patients with high fever. Commercial penicillin is now carefully treated to eliminate any such dangerous proteins.

In the manufacture of penicillin the chief difficulty is to insure sterility of the cultures. It is a strange thing that this antibiotic which is a potent killer of numerous bacteria is itself killed by some of the bacteria which it cannot conquer. Therefore, it must be cultured under the most rigid aseptic conditions. The ordinary colon bacillus which is not inhibited by penicillin produces such a potent enzyme for the destruction of penicillin that it is called penicillinase by Abraham and Chain (18).

The optimum temperature for the growth of *Penicillium* is 24° C. The source of the carbon for its nutrition is lactose and the source of its nitrogen is sodium nitrate. Zinc ions seem also to be necessary for its production. The composition of corn-steep water which so greatly increases the yield of penicillin is not entirely known but in a recent article by Gregory Schwartzman in *Science* he showed that if cellophane is suspended in the culture medium, it also increases the yield of penicillin. It may be then that corn-steep water, a by-product in making glucose and corn-starch may contain a cellulose derivative similar to cellophane.

Since the *Penicillium* mold is an aerobe, it must have an adequate supply of oxygen. The aeration of the culture then had to be solved. Three devices were used to secure aeration. One was the surface method. This older method was to grow the mold in flat half-gallon bottles or flasks. The films of mold were floating in culture medium not more than 2 cm. deep. Aeration was thus by simple diffusion. In this method contamination of the bottles with foreign organisms was a great danger. A second method was the bran method. In this method coarse bran was spread in large trays and the mold culture was sprayed on the bran and after the usual growth period the penicillin was extracted from the mass. The third method is the Submerged Culture Method and all the large plants now use this procedure. The mold is grown in high cylindrical tanks each holding 1,000 gallons. Aeration is brought about by a vigorous bubbling of sterile air through the mass in the culture tanks. The yield per milliliter by this method is slightly less than culture in bottles. The only problem in the Submerged Method is to insure the complete sterilization of the large volumes of air needed to be blown

through the tanks. By 1945 the combined output of twenty-one companies making penicillin was 200 billion units per month or about nine pounds of penicillin per day.

The method of extracting the penicillin from the culture medium is as follows: The pH of the fluid is adjusted to 1.9 or 2 with phosphoric acid. It is then agitated with a solvent that is immiscible with water, such as amyl acetate or chloroform. After separation of this solvent the aqueous part is discarded. The amyl acetate containing the penicillin in acid form is shaken up with a little water containing sodium bicarbonate. This converts the penicillin to the sodium salt. The sodium salt of penicillin is further purified by adsorption on columns of alumina or with activated charcoal. It is now chemically usable but it is considered desirable to remove traces of amyl nitrate that might be left, so the sodium salt is usually filtered through Seitz bacterial filters and dried by sublimation from a frozen state under reduced pressure. The product is a yellow powder which is quite stable when perfectly dry and is best kept in a refrigerator. When needed for use it is merely dissolved in water or in a normal saline solution.

ASSAY METHODS FOR PENICILLIN

The strength or concentration of penicillin is measured by a bioassay method which depends on its antibiotic properties. The unit was devised by Professor H. W. Florey and M. A. Jennings (19) and is called the Oxford unit. It was published in 1942 in the British Journal of Experimental Pathology. The Oxford unit is now the standard for this drug. It is the amount of penicillin dissolved in 50 ml. of meat extract broth which will just inhibit the growth of a test strain of *Staphylococcus aureus*. Thus a preparation of penicillin which will just inhibit the test strain of the coccus contains one unit. With a dilution of 1 : 50,000 the technic of making the assay is by the cup method. The standard coccus No. 209 is grown on a petri dish and in it are set five small porcelain cups. In two of these cups is placed a standardized solution of penicillin of one unit strength per milliliter. Into the other three cups is placed some of the penicillin solution whose assay is being made. The plates are incubated over night. In the morning the examination of the clear zones around each cup where the penicillin has penetrated the agar and killed the coccus is made. Comparisons of the widths of the zones of the known strength with those of the unknown is the basis of the assay. Pure crystallin penicillin has a standard assigned strength of 1650 units per milliliter or stated in another way 0.6 grams of penicillin has an antibiotic activity of one million Oxford units. The uses for which penicillin is indicated will be given later.

The story of the other important antibiotic, *streptomycin*, began with the search for some antagonist to the gram-negative bacteria. Penicillin combats effectively against the gram-positive forms. But against the gram-negative pathogens of typhoid, colon bacillus infections, dysentery, undulant fever and tularemia and the acid fast bacteria such as *Mycobacterium* which causes tuberculosis, it is impotent. In 1939 the Department of Microbiology at Rutgers University began to study antibioltic substances and under the leadership of Dr. Selman A. Waksman (20) they isolated seven compounds. These are: 1, *actinomycin*, which was found to be highly toxic to animals; 2, *clavicin*, which was active against gram-negative bacteria but also toxic; 3, *fumigacin*, not so toxic but also not very active; 4, *chaetomin*, which was non-toxic but also ineffective in animals; 5, *micromonosporin*, which held little promise; 6, *streptothricin*, which was effective as an antibiotic but still toxic to animals; and finally 7, *streptomycin*. This last substance was isolated from an actinomycete. These are microbial organisms between bacteria and fungi which form thread-like growths in the soil. The organism is named *Streptomyces griseus* and was isolated twenty-eight years previously, (21) as an organism living in soils and composts. In 1939 Dr. Waksman and his colleagues secured two cultures of this actinomycete. One from a heavily manured field near Rutgers

and the other from the throat of a chicken. The *Streptomyces* was grown on a liquid medium which, to produce the best results, contained the elements of meat extract. The carbon nutrients could be supplied by starch and glycerol and the nitrogen from casein and sodium nitrate. The final culture medium adopted was composed of glucose, peptone, meat extract, sodium chloride and water with a pH of 6.5–7. If a culture is shaken, the growth is more rapid, a maximum growth being attained in two to three days. In stationary cultures the maximum growth requires nine days. In either method the culture medium becomes alkaline. The strains of *Streptomyces* are not quite constant but in general they undergo very little variation under cultural conditions. For the most part highly potent strains retain their potency and poor strains remain weak producers of streptomycin. The streptomycin is formed equally well in stationary or submerged cultures. Streptomycin is water soluble and may be easily isolated from the culture medium.

When the broth culture has attained its maximum antibiotic activity, the incubation is terminated and the mass is centrifuged or filtered to separate the broth. The selection of the filter is important since streptomycin is rapidly adsorbed on various filters. The filtered fluid is alkaline and is next treated with activated charcoal which completely adsorbs the streptomycin. The charcoal adsorbate is centrifuged or filtered off and washed with alcohol to remove impurities. Then it is treated with dilute acid aclohol to bring the streptomycin into solution. The acid alcohol solution is then neutralized and filtered to remove any final impurities that have precipitated out. The resulting solution is concentrated by adding ten volumes of ether which takes up the alcohol and leaves the streptomycin as a yellow, brownish or reddish aqueous concentrate. By precipitation with acetone or by dessication in a vacuum, solid streptomycin is obtained. A more pure form can be obtained by the addition of methyl orange, which is the sodium salt of helianthine. The resultant crystals are streptomycin-helianthate from which soluble salts of streptomycin can be obtained. Another method of obtaining purer salts is by precipitation of the charcoal adsorbate with phosphotungstic acid and picric acid. Reinecke salt is added to the aqueous solution which forms crystalline precipitates and by fractional distillation pure streptomycin reineckate is obtained. These are then converted to either the hydrochloride or the sulfate of streptomycin.

STREPTOMYCIN ASSAY AND UNIT STRENGTH

The assay of streptomycin may be done in various ways as the agar dilution streak, or the agar cup method or the turbidimetric method. The original unit was determined by the amount of streptomycin in one milliliter of nutrient broth which will just inhibit a given strain of *Escherichia coli*. This was called the S unit. Since this unit is so small, it was found to be more convenient to use the antibiotic in one *liter* of broth instead of a milliliter, hence this unit is called the L unit. In dealing with the pure crystalline material it was found that 1,000 L units or 1,000,000 S units could be approximated closely by dry weight, so now the G unit is used which is one microgram of dry crystalline streptomycin. In a comparison of the Oxford units of penicillin and the G units of streptomycin it must be remembered that they have no similar basis. In dry weights of the two antibiotics one streptomycin unit is one microgram whereas an Oxford unit of penicillin weighs only 0.6 microgram. The bacteria used in the two assays are also quite different, *Staphylococcus aureus* being gram-positive and *Escherichia coli* being gram-negative.

In contrast to penicillin, streptomycin is remarkably stable both chemically and biologically. The latter can be stored in a refrigerator for six months without any loss of potency; also no other organisms destroy it as they do penicillin.

Bacitracin, which was discovered in 1945 by Drs. B. A. Johnson, Herbert Anker, and Frank L. Meleney at Columbia University's College of Physicians, appeared in the broth on which was cultured organisms derived from wounds.

It is formed by a type of *Bacillus subtilis*. The filtrate is extracted with butanol, concentrated by steam distillation and precipitated as a grayish white powder. It is neutral, and unlike gramicidin or subtilin, cannot be extracted with ether, chloroform or acetone. It is water soluble and non-toxic. It is active in animals and human infections against gram-positive organisms as *Streptococcus, Stephylococcus* and gas-gangrene due to *Clostridium*. It is used clinically by its discoverers but is not yet standardized nor commercially available.

CLINICAL USES FOR PENICILLIN AND STREPTOMYCIN

During the latter part of the recent war Dr. Chester B. Keffer, Chairman of the Committee on Chemotherapeutic and Other Agents, of the National Research Council published a report on analyses of thousands of cases treated with penicillin. This report was issued by the Office of Civilian Distribution of Penicillin.

In the discussion of the uses for penicillin as a drug the application was divided into four classes or groups:

Group I shows that penicillin is indicated for all infections due to *Staphylococcus*, as carbuncles, meningitis, sinusitis, pneumonia, wound infections and osteomyelitis; also gas gangrene due to *Clostridia;* all infections due to *Streptococcus*, such as cellulitis, mastoiditis, meningitis, peritonitis or pneumonia; all anaerobic *Streptococcus* infections as puerperal sepsis; all infections due to *Pneumococcus* in the pleura, meninges or endocardium; and all cases of sulfa-resistant *Pneumococci;* and finally all infections due to gonococci.

Group II shows that penicillin is effective but its potency is not yet clearly established in cases of syphilis, actinomycosis and bacterial endocarditis.

Group III shows that penicillin is of questionable value in mixed infections in which gram-negative organisms predominate, such as ruptured appendix, liver abscesses, urinary tract infections and Rat-bite fever due to *Streptobacillus moniliformis.*

Group IV shows that penicillin is contra-indicated because it is ineffective in all gram-negative bacillary infections of typhoid, colon bacillus, dysentery, influenza, undulant fever and tularemia. Also it is impotent in tuberculosis, acute rheumatic fever, lupus, infectious mononucleosis, pemphigus, Hodgkin's Disease, acute and chronic leukemia, ulcerative colitis, coccidiomycosis, poliomyelitis, all virus diseases and cancer.

This rather complete survey indicates the clear cut benefits of its use as well as the large area of diseases in which it is of no value at all.

Now to consider the indications for streptomycin therapy. No complete summary has yet been issued for the uses of this drug since much work is still being done upon it. In general it can be stated with reasonable assurance that streptomycin is indicated in most cases of infection that are resistant or wholly untouched by penicillin, sulfa drugs or serum therapy. According to Greey (22) of Toronto University, streptomycin is effective in chronic infections of the urinary tract due to *Escherichia coli, Aerobacter aerogenes* or *Pseudomonas aeruginosa.* In four to eight hours after treatment with streptomycin given intromuscularly the urine was negative for these organisms. It is also indicated for infections of the intestine due to typhoid and related organisms, via., *Eberthella, Salmonella* and possibly *Shigella* of the dysentery organisms. Flippin (23) of the University of Pennsylvania states that patients with twenty-three million *Salmonella* (paratyphoid) bacterial count in the stools gave negative stools after four days therapy with one gram of streptomycin given orally per day.

This drug is also indicated in systemic diseases due to *Brucella* infections such as brucellosis or undulant fever This was recently communicated by H. A. Reimann (24).

Infections due to *Klebsiella* or Friedländer's bacillus causing forms of pneumonia and a venereal disease are definitely cured by streptomycin. A report by Heilman (25) of the Mayo Clinic last year states that patients with *Klebsiella* infections of the respiratory tract showed negative sputa in a short time.

Infections due to *Pasteurella tularensis*, the organism of Rabbit Fever or tularemia, have been treated by Foshay (26) of Cincinnati University. He reports that a patient who on the eighth day after the onset of the disease received streptomycin therapy was sent home cured on the seventeenth day after admission to the hospital, although the average duration of tularemia is three to nine months.

Perhaps the most striking application of the use of streptomycin, although still in the experimental stage, is the treatment of tuberculosis. Each day at present new reports are coming out of the trials of this drug against *Mycobacterium tuberculosis*. Feldman and Hinshaw (27) of the Mayo Clinic have treated twenty-two tuberculosis patients with streptomycin for nine months. They report that it has a suppressive effect on the disease and that its progress is temporarily inhibited. It is to be hoped that research on the use of streptomycin in combating this great white plague of civilization will continue and that it will prove to be a trustworthy chemical weapon against this disease. Only last week (April 6, 1947) U. S. Surgeon General Thomas Parran appeared before the Senate Appropriations Committee and urged that it approve a fund of one million dollars for streptomycin research against tuberculosis. Dr. Herman E. Hilleboe, in charge of the research, told a committee in the Senate that while the original cost of streptomycin of twenty dollars per gram is now reduced to three dollars per gram, even yet at the present rate it requires ten to twelve dollars' worth of the drug daily for each patient.

CLINICAL ADMINISTRATION OF ANTIBIOTICS

Penicillin may be administered intramuscularly, intravenously or into body cavities such as the pleura and the sub-arachnoid space, or locally. Some of its salts are irritating to tissues, notably the sodium salt. A constant intravenous drip injection of thirty to forty drops per minute into a vein has been found most effective in reducing acute infections. It is excreted very rapidly in the urine and two hours after an initial dose is given, it is impossible to detect it in the blood. About 40,000 to 120,000 units per day bring susceptible infections rapidly under control.

Streptomycin also may be given by intravenous or intramuscular injection. It likewise is rapidly excreted by the kidneys but by frequent injections a constant therapeutic level may readily be maintained in the blood. By oral administration streptomycin does not easily get into the blood stream and so its effectiveness is not gained by administrations through the mouth. But in the intestine it is not destroyed and its presence can easily be detected in the stools where it exerts a markedly repressive effect on the normal bacterial population of the intestine. Streptomycin given by injection may be quite painful and one symptom of its use over a period is the production of tinnitus or a ringing sensation in the ears. In some cases this is extended to deafness for a longer or shorter time following its employment.

In concluding this discussion of antibiosis I am sure that we all agree that the present decade which has made possible the applications of these miracle-working antibiotic substances has placed in the hands of man one of the most powerful weapons against disease and that the life and health of millions will be preserved by the patient studies of men like Fleming and Waksman. They will be rated in history with the great benefactors of our race.

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